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Testing of Intravenous Hemostatic Agents in a Novel Swine Model of Bleeding: Preliminary Results with FXa-PCPS

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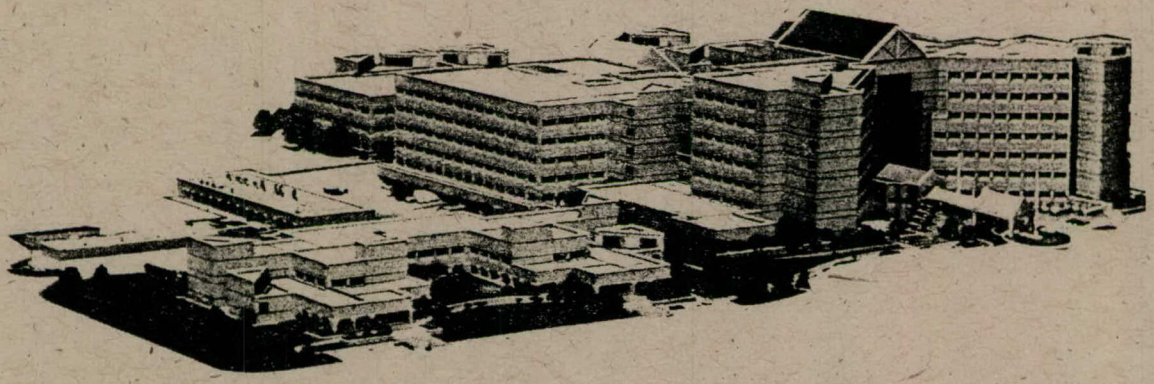

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14. ABSTRACT

Finding an intravenous treatment that could assist endogenous clotting mechanisms is a major mission for military researchers. Herein, we report preliminary data on the efficacy of mixtures of highly purified blood coagulation factors (FVIIa and FXa) and phospholipids vesicles (PCPS) to reduce traumatic bleeding. A novel swine model was developed in which 1) bleeding times (BT) and coagulation function could be ascertained following multiple doses of drug administration; and, 2) an exsanguinating injury could subsequently be performed in the same animal, yielding screening information as to the effects of drug pretreatment of blood loss and survival. FXa-PCPS failed to reduce BT or enhance coagulation function and a dose-dependent hypotensive response. FVIIa-PCPS yielded no data suggestive of the profound potentiation of FVIIa activity by PCPS observed in previous in vitro testing. Further study of these drugs for the use of traumatic hemorrhage is not warranted.

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Testing of Intravenous Hemostatic Agents in a Novel Swine Model of Bleeding: Preliminary Results with FXa-PCPS and FVIIa-PCPS

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ABSTRACT

Because uncontrolled hemorrhage is a major source of battlefield mortality, finding an intravenous treatment that could assist endogenous clotting mechanisms is a major mission for military researchers. Herein, we report preliminary data on the efficacy of mixtures of highly purified blood coagulation factors (FVIIa and FXa) and phospholipids vesicles (PCPS) to reduce traumatic bleeding. For this initial screening, we developed a novel swine model in which 1) bleeding times (BT) and coagulation function could be ascertained following multiple doses of drug administration; and, 2) an exsanguinating injury could subsequently be performed in the same animal, yielding screening information as to the effects of drug pretreatment on blood loss and survival. Transection of small mesenteric arteries and veins allowed for multiple and reproducible BT measures that correlated with coagulation function. Subsequent excision of defined areas of the liver produced bleeding predominantly from small vessels (<2 mm diameter) and parenchyma, while still resulting in 60% mortality without the use of either heparinization or aggressive fluid infusion. FXa-PCPS failed to reduce BT or enhance coagulation function; in fact, our preliminary results demonstrate a prolongation of BT, a diminution of coagulation function and a dose-dependent hypotensive response. FVIIa-PCPS yielded no data suggestive of the profound potentiation of FVIIa activity by PCPS observed in previous *in vitro* testing. Data from these preliminary experiments suggest that further study of these drugs for the use of traumatic hemorrhage is not warranted.

INTRODUCTION

Hemorrhage is the leading cause of death from wounds on the battlefield, accounting for over 50% of those deaths [1]. Hemorrhage is also the second leading cause of death in civilian trauma [2]. The proportion of deaths as a result of combat injuries increases with increasing evacuation time [1, 3]. For example, the rate of death prior to evacuation increases from 20% with a two-hour evacuation, to 26% with a six-hour evacuation, to 32% with a 24-hour evacuation. Of the increased deaths due to delayed evacuation, 62% are the result of hemorrhage [1]. This represents a group of casualties who bleed from wounds that are not immediately fatal. Approximately 80% of hemorrhagic combat deaths are from wounds that are not compressible (not accessible for manual pressure). Currently, there is no method available forward of the operating table that can provide hemorrhage control for non-compressible hemorrhage. An intravenous drug that could augment the body's innate clotting mechanisms could therefore provide an additional tool for hemorrhage control when surgical intervention is not possible.

The concept of pharmacologic hemostasis is not new. Drugs have been used in the treatment of bleeding complications for over 30 years [4]. In spite of the common use of pharmacologic methods for decreasing blood loss in elective surgeries and specific coagulopathies in which large blood losses are expected, the potential for use to aid hemostasis during traumatic hemorrhage has not been adequately studied.

A relatively newer drug (technically a biologic by FDA classification), recombinant factor VIIa (rFVIIa), is an enzyme that has been used very effectively in hemophilia patients for controlling muscle, joint, mucocutaneous, vascular, urinary tract, and gastrointestinal hemorrhage [5-8] and during surgical procedures [5, 6]. This drug

appears safe, with only rare reports of thrombotic or immunological complications [5-10]. It does not appear that rFVIIa induces a generalized hypercoagulability but that its action is limited to the site of injury [5, 11-13]. Preliminary results in both animal and human subjects indicate that rVIIa may also play a beneficial role in the treatment of hemorrhage control in cases of trauma [13-19], although this is not a universal finding [20-23].

Both Factors VIIa and Xa play crucial roles in the extrinsic cascade of blood coagulation. Circulating factor VII/VIIa forms a complex with tissue factor (TF) exposed at the site of injury and initiates the coagulation cascade by activating factors X and IX, ultimately leading to the generation of thrombin. Prothrombin conversion to thrombin proceeds through a prothrombinase complex (prothrombin, factor Va, calcium, and phospholipid). Thrombin is the final product in the coagulation cascade responsible for converting the soluble protein, fibrinogen, to its gel form fibrin (fibrin clot).

Thrombin formation *in vivo* proceeds via a series of enzymatic reactions presumed to occur on membrane surfaces provided primarily by platelets [24]. *In vitro* studies primarily utilize artificial phospholipid vesicles (PCPS) as a surrogate membrane surface to study the process of blood coagulation. It has been demonstrated that PCPS at 1 to 2 μmol/L is equivalent to platelets at physiological concentrations (2×10^8/mL) in supporting thrombin generation [25]. However, platelets (and phospholipid) at physiological concentrations do not saturate the reactions of blood coagulation [26-28]. In theory, providing an extra surface for the reactions of blood coagulation to occur should increase the quantity and efficiency of the enzymatic complexes of blood coagulation. Addition of phospholipid (PCPS) at higher saturating concentrations should

therefore substantially affect the enzymatic efficiency of the prothrombinase complex. Addition of factor VIIa would create more VIIa/TF complex, which in turn would generate more factor Xa. Increased levels of factor Xa along with additional surface in the form of PCPS would promote the formation of more and more efficient prothrombinase complexes, subsequently increasing thrombin generation and ultimately increasing clot formation. These considerations provided the impetus for our hypothesis: addition of a saturating surface (PCPS) in combination with either factor Xa or factor VIIa will augment hemostasis by forming more as well as more efficient enzyme complexes to produce more thrombin, thereby providing a means of hemorrhage control.

Butenas and his colleagues have recently demonstrated the influence of rVIIa and PCPS lipid vesicles on thrombin generation and clot formation *in vitro* [29, 30]. Thrombin generation was evaluated in terms of its rate of formation as well as total quantity of thrombin produced. The parameters used to assess clot formation included overall time to clot, clot weight, fibrinogen depletion, and generation of fibrinopeptide A. Results from this work indicate that the contribution of rVIIa to tissue factor-initiated coagulation in normal blood as well as in induced hemophilic blood is improved with regards to thrombin generation and clot formation by the presence of phospholipids (i.e., PCPS) and rVIIa as compared to the addition of either of the components alone. The combined effect of both rVIIa and PCPS can increase the concentration of the factor VIIa/TF complex and subsequently provide phospholipid surface for additional enzymatic complexes of blood coagulation. These findings supported our hypothesis and warranted *in vivo* investigation into the hemostatic potential of VIIa/PCPS mixtures as a treatment for non-compressible hemorrhage.

Factor Xa in conjunction with PCPS also appears quite promising as a potential drug candidate to be evaluated for the control of acute non-compressible hemorrhage. It has been demonstrated that the *in vivo* thrombogenicity of prothrombin complex concentrates is highly correlated with their individual content of coagulant-active phospholipid [31]. Thrombogenicity was measured *in vivo* in a stasis model in rabbits where thrombogenicity is expressed as the number of animals with thrombi as a percentage of the total group tested [31]. The phospholipid component alone was nonthrombogenic but required the presence of Factor Xa to produce thrombi. At high doses, the latter was thrombogenic alone but its potency was drastically increased in the presence of small amounts of coagulant-active phospholipid. It was suggested that the combination of these two components accounted for the thrombogenicity associated with the use of prothrombin complex concentrates and evidence was presented that this thrombogenic effect could be mimicked by a combination of highly purified Factor Xa and PCPS lipid vesicles. This study also provided significant evidence correlating thrombin generation (and clot formation) to the presence of additional phospholipids and purified coagulation factors (i.e., Factor Xa).

In combination with the previous findings, patent claims (U.S. Pat. No. 4,610,880 / 4,536,392 / 4,721,618) held by Haematologic Technologies provided a further foundation for the research reported herein. In short, these claims relate to the control of bleeding in mammals (i.e., dogs). Specifically, this invention relates to the reduction of cuticle bleeding times in both hemophilic and normal dogs when administration of a synergistic mixture of PCPS and Factor Xa were used. In normal dogs, bleeding stops abruptly (6.0 ± 3.7 min post-injury whereas in hemophilic animals bleeding may stop

transiently but always restarts. Infusion of Xa/PCPS significantly accelerated the hemostatic process by stopping the bleeding in normal subjects (30 sec) and was effective at correcting the bleeding times (equivalent to normal) in subjects with a hemophilic defect [32, 33]. Furthermore, infusion of Xa/PCPS accelerated and normalized the formation of a hemostatic plug in normal and hemophilic dogs, respectively [33].

The objectives of this pilot work were therefore two-fold. First, we sought to develop a new animal model that would be useful in screening intravascular agents for their ability to enhance coagulation function and reduce bleeding. We successfully developed an animal model in which 1) bleeding time and in vitro hemostatic parameters could be measured multiple times; and, 2) an exsanguinating liver injury featuring primarily parenchymal and small vessel bleeding could be subsequently created in the same animal. Second, we determined whether infusion of either FXa-PCPS or FVIIa-PCPS (with lower doses of FVIIa than previously used in experimental swine studies) would reduce blood loss in this new model. Because of lack of efficacy and the presence of adverse side effects with these drugs at the concentrations available, the decision was made by the investigators that these drugs were not worthy of further consideration for use in traumatic bleeding. Therefore, only preliminary results are presented herein.

METHODS AND MATERIALS

Animals and Instrumentation

Crossbred commercial swine weighing 38.4 ± 0.5 kg (mean ± SEM) were used in this study. Animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. This study was

approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research, Fort Sam Houston, TX. Animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1996).

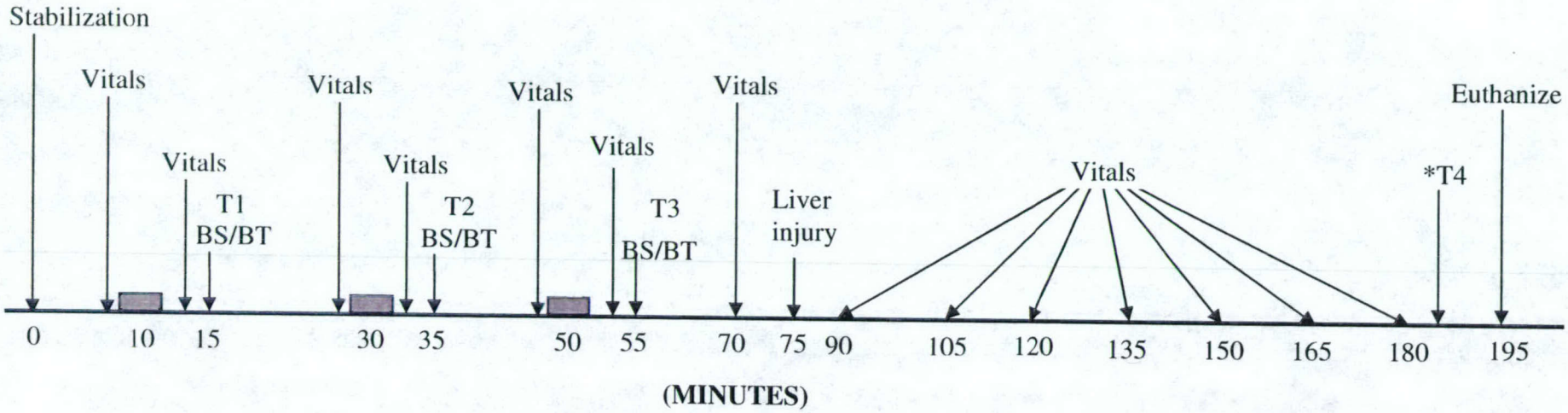
Swine were fasted for 24 to 36 hours before the procedure, with water continuously available. Animals were sedated using glycopyrrolate and Telazol^{R}. Buprenorphine (0.9 mg, i.m.) was administered for analgesia. They were then and anesthetized (1-4% isoflurane in room air) and intubated using a closed circuit system with mechanical ventilation. Infusion catheters were placed occlusively in a femoral vein and a jugular vein. Maintenance fluid (lactated Ringer's; 5 ml/kg/hr) was continuously infused during the experiment. An 8.5 Fr catheter introducer was shortened to 3 cm and placed occlusively in a femoral artery for blood sampling. A Paratrend 7+ Multiparameter Sensor-catheter (Diametrics Medical Inc., Roseville, MN) was placed occlusively into a carotid artery and was attached to a Trendcare TCM 7000 blood gas monitoring system (Diametrics Medical Inc., Roseville, MN) for continuous monitoring of body temperature and blood pH. A port in the Paratrend 7+ catheter was coupled to a continuous data collection system (MicroMed®, Louisville, KY) for monitoring blood pressure and heart rate. Laparotomy, splenectomy, and cystotomy were performed in each pig. To compensate for removal of the spleen, each animal was infused with lactated Ringer's solution at a volume equivalent to three times the spleen weight. Animals were stabilized for 20 minutes at a body temperature of 38.5 to 39.5°C, a blood pH of 7.35 to 7.45, and a mean arterial pressure (MAP) between 50 and 80 mmHg.

Mesenteric Bleeding Times

Following stabilization, a distance of 30 cm was measured from the ileo-cecal junction along the ileum, and a second mark was made 15 cm further from this point. A U-shaped plastic tube (24 cm long x 4 cm diameter) was laid under this 15 cm section of mesentery and 3 small arteries with accompanying veins were identified within this area (Figure 1). These vessels were approximately 1 mm in diameter. Each vessel was sharply transected with iris scissors and time to cessation of bleeding was measured, with 10 minutes (600 seconds) being arbitrarily chosen as the maximum BT possible. BT was then taken as an average across these triplicates, with elimination of a single value when the CV exceeded 10%. For each pig (n=18), BT was repeated along the mesentery twice more at 20 cm and 20 min intervals, as shown in Figure 2. Data from these pigs were subsequently analyzed to determine the reproducibility of this procedure in regards to time after laparotomy and location within the mesentery. To determine the ability of this BT measurement to reflect coagulation status, BT was measured in an additional 3 pigs after infusion of heparin (50, 75, and 100 IU/kg) and again after multiple doses of protamine sulfate (0.5 mg/kg) to reverse the effects of heparin.

Figure 1. Performance of mesenteric BT procedure in an anesthetized pig.

Figure 2. Timeline for Drug Screening Experiment



Drug Infusion
BS Arterial Blood Sample
BT Bleeding Time
* Taken before death (MAP≤20)

Injury Phase

Following completion of BT determinations, a major uncontrolled hemorrhage was induced in 10 pigs. The peritoneal cavity was first suctioned and laparotomy sponges were positioned under the left medial and lateral liver lobes and within the gutters of the abdominal cavity. These sponges were clamped together for easy and immediate egress. Subsequently, the distances between the entry of the inferior vena cava into the liver and the caudal edges of both the left medial and left lateral liver lobes were measured. Each lobe was then loosely clamped approximately 45% of the distance from the caudal liver edge to the inferior vena cava. These sections were sharply cut to remove the distal aspects of each lobe. The clamps were then removed and the liver was allowed to bleed freely (Figure 3a). All sponges were swiftly removed 30 seconds after excision and the abdomen was temporarily closed. Infusion of maintenance fluid was discontinued and no resuscitation fluids were provided. Animals were continuously monitored until death or for 2 hours, at which point surviving animals were euthanized. After death, intraperitoneal blood was measured using suction into pre-weighed canisters and pre-weighed gauze sponges. Additionally, the number and size of transected vessels were measured on the excised portions of the liver (Figure 3b) and confirmed post-death on the remaining liver sections. Vessels were arbitrarily classified as being small (diameter $\leq$ 2mm), intermediate (2 mm>diameter $\leq$ 4mm) or large (diameter > 4 mm).

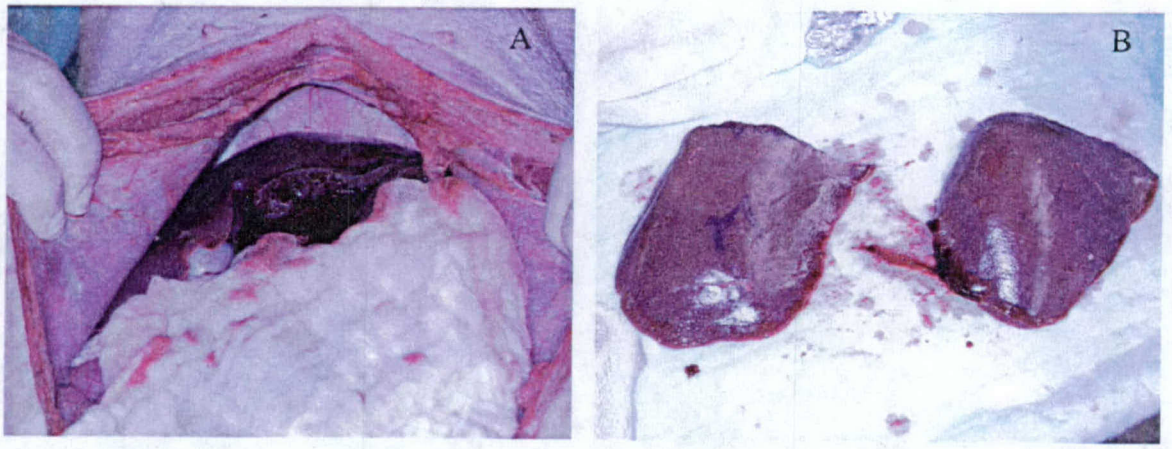


Figure 3. Panel A, freely bleeding liver following excision of approximately 45% of the left lateral and left medial lobes (Panel B).

Drug Treatments

Purified human FVIIa and human FXa, as well as PCPS were provided as lyophilized powders by investigators at Haematologic Technologies, Inc. (RHD and RJJ). Human FVIIa and Xa were obtained by fractionating plasma from normal human donors to obtain the precursor zymogen factors VII and X; these were subsequently activated to yield FVIIa and Xa. The proteins were prepared by lyophilization in 20 mM HEPES, 150 mM NaCl, 1.0% polyethylene glycol (PEG), and 0.1% HAS at a pH of 7.4. Prior to experimentation, reconstitution was accomplished by adding sterile 2.5 mM $CaCl_2$ in water. Following reconstitution, FXa or FVIIa was mixed with PCPS (reconstituted in sterile water) and diluted with a buffer solution (tris-buffered saline (TBS), 1% polyethylene glycol (PEG), pH 7.4). Our initial pig (#20) received doses of FXa-PCPS in a total volume of 8 ml; this infusion resulted in profound hypotension following the first dose and immediate death following the second. Following consultation with Dr. Michael Nesheim (who performed many of the original experiments), it was decided that the drug must be infused in larger volumes (1 ml/kg body weight); this was done in all

subsequent experiments. As these were preliminary screening experiments, a variety of doses were used (Table 1). Doses were chosen based on previous in vitro [29] and in vivo [31-33] experiments and in consultation with Dr. Nesheim. In each experiment, the initial infusion of drug consisted of an identical volume of LR (at T1) to serve as control; all T1 values reported in the tables that follow are therefore baseline levels.

Table 1. Drug treatments administered to each pig.

PIG #	Date	Treatment	Low Dose (per kg) (pmoles protein/nmoles PCPS)	High Dose (per kg) (pmoles protein/nmoles PCPS)
20*	25 Oct 04	FXa/PCPS	5.22 / 14	25.4 / 40
21	27 Oct 04	FXa/PCPS	5.22 / 14	25.4 / 40
54	15 Nov 04	FXa/PCPS	1.5 / 2.31	7.5 / 11.54
62	22 Nov 04	FXa/PCPS	2/3.86	5/7.73
102	24 Jan 05	FXa/PCPS	2.5/4.0	25.5/40
101	26 Jan 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
103	2 Feb 05	FXa/PCPS	2.5/4.0	25.5/40
118	7 Feb 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
121	9 Feb 05	FXa/PCPS	2.5/4.0[b]	25.5/40[a]
24	3 Nov 04	FVIIa/PCPS	9.0 / 14	25.4 / 40
55	15 Nov 04	FVIIa/PCPS	9.0 / 56	25.5 / 280
53	22 Nov 04	FVIIa/PCPS	350 / 538.5	373.8 / 569.2#
25	3 Nov 04	PCPS	14	40

*Doses given in 8 ml total volume. In all subsequent experiments, doses given in 1 mL/kg total volume.
#Values reflect an attempt to achieve a final cumulative dose of 700 pmoles VIIa using a half-life of 147 min for factor VIIa. Drug administered every 20 min.
[a,b]High dose given first followed by low dose

Blood Sampling

Blood samples were collected by inserting a 20 cm single-use catheter made from Tygon® tubing (I.D. 0.9 mm; Saint Govaine Performance, Akron OH) into the self-sealing port of the catheter introducer and gently withdrawing the blood to minimize

shear-induced platelet activation. The first 3 ml of blood was discarded. Except for thromboelastography (TEG) and activated coagulation time (ACT) samples, blood was anticoagulated with 3.2% sodium citrate at 1 part citrate solution in 9 parts blood.

Standard Laboratory Procedures

Hematocrit (Hct), hemoglobin (Hb), and platelet (PLT) counts were performed as direct measurements using the ABX Pentra 120 hematology analyzer (ABX Diagnostics, Inc., Irvine, CA). ACT was performed using the Hemochron Response (International Technidyne Corp., Edison, NJ), according to manufacturer's instructions. FVII or FX activity was measured with a one-stage clotting assay using an automated coagulation analyzer (Dade Behring BCS system, Marburg, Germany). For the assay, normal and FVII or FX deficient human plasma standards and rabbit brain thromboplastin reagent provided by the manufacturer were used. The BCS system methodology reports results in percent activity. Samples were diluted as necessary according to manufacturer specifications. Standard prothrombin time (PT; using commercial rabbit brain reagent), activated partial thromboplastin time (aPTT), and fibrinogen concentrations were determined at 37°C using an automated coagulation analyzer (Dade Behring BCS system, Marburg, Germany) according to manufacturer specifications. Thrombin-antithrombin III (TAT) concentrations were quantitated using the Enzygnost TAT micro enzyme immunoassay (Dade Behring, Marburg,Germany), which has previously been demonstrated to cross-react with porcine TAT [34].

Thromboelastography (TEG)

TEG monitors changes in the viscoelastic properties of a forming clot (see [23] for a complete description). Briefly, TEG yields various standard parameters, including

reaction time (R), coagulation time (K), α-angle, maximum amplitude (MA), and time to reach MA (MA). R reflects the period of latency from the start time to initial clot formation. K reflects the time from R until a standardized level of clot firmness is reached (amplitude = 20 mm). The α-angle is a measure of the kinetics of clot development. MA is the maximum amplitude attained and reflects the maximum firmness of the clot. Two additional TEG parameters were calculated, maximum velocity (MaxVel), and time to MaxVel (tMaxVel).

TEG was performed in the final five FXa-PCPS pigs using the model 5000 TEG (Haemoscope, Skokie, IL) as previously described [23]. Tissue factor (pig thromboplastin) was used as the agonist, with the concentration being a dilution of the original stock solution that yielded a 3 second R value time [23]. TEG was simultaneously performed using 50 μl of saline as a control to allow confirmation that the clotting observed in response to the agonist was predominantly due to agonist activity. Unaltered whole blood (300 μl) was delivered to each cup within one minute of collection to initiate clotting. TEG was performed in triplicate. The single value for each TEG parameter was derived by averaging, with elimination of a single value when the CV exceeded 10%.

Pathology

Samples from kidney, lung, mesentery, heart, and skeletal muscle were collected within 10 minutes post-mortem and fixed in formalin. All samples were embedded in paraffin, sectioned, and stained using hematoxylin and eosin, Masson's Trichrome, and phosphotungsten acid hematoxylin. Tissues were examined under light microscopy by a

board-certified veterinary pathologist for evidence of disseminated intravascular coagulation (DIC) and microthrombi formation.

Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS) statistical package [35]. Survival data were analyzed using PROC FREQ and associated Fisher's Exact test. Differences among numbers of sizes of blood vessels cut were also examined using PROC FREQ with the Chi Square test. Analysis of all survival time data was conducted using the PROC LIFETEST procedure of SAS with associated Log-Rank nonparametric test. Blood loss and the percentages of lateral and medical lobes cut were analyzed using a single-way analysis of variance with pigs as a random factor (PROC GLM). All data were tested for homogeneity of variance (PROC ANOVA with associated Levene's test) and normality of distribution (PROC Univariate Normal with associated Kolmogorov-Smirnov test). Data were transformed where necessary to meet assumptions of ANOVA. All data are presented as arithmetic means ± SEM.

RESULTS

Development of Model

To determine whether mesenteric BT reflected coagulation status, BT was measured in 3 pigs after: 1) no treatment; 2) 50, 75, and 100 IU heparin/kg body weight; and, 3) three administrations of protamine sulfate (0.5 mg/kg). *In vivo* BT increased with increasing doses of heparin, and then decreased after multiple administrations of protamine ($p=0.042$; Figure 4). ACT varied in a similar manner ($p=0.06$; Figure 4).

There was a highly significant correlation between in vivo BT and ACT (r=0.88; p=0.003).

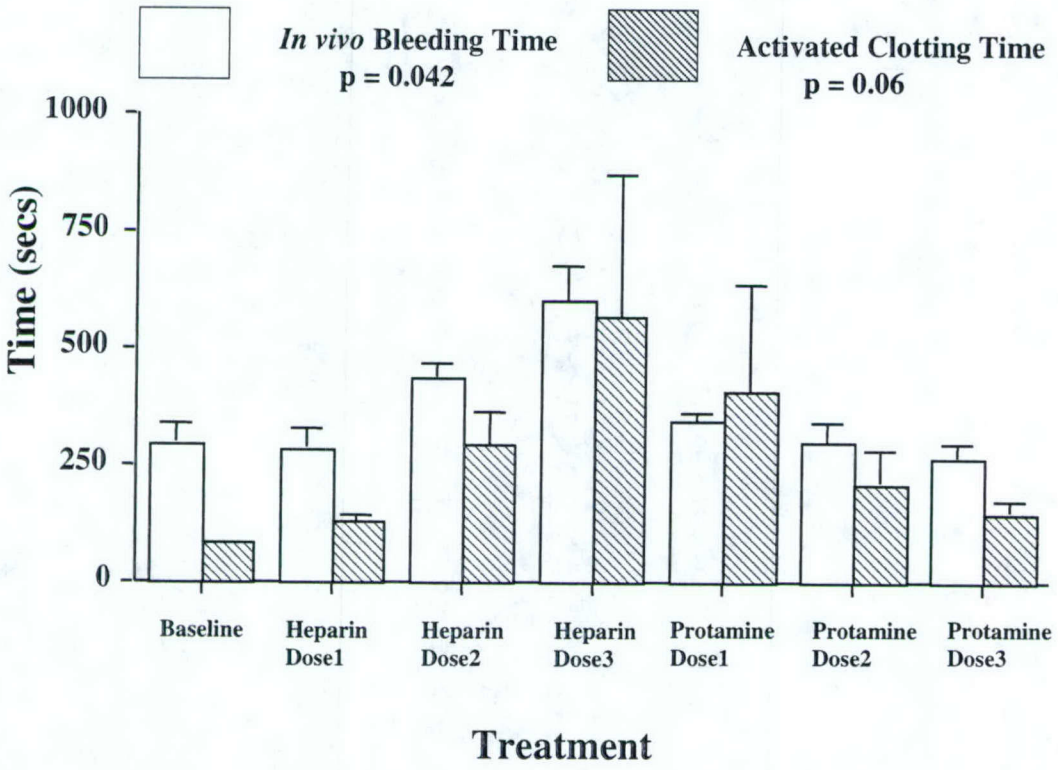


Figure 4. In vivo bleeding time (BT) and activated clotting time (ACT) following doses of heparin and protamine (n=3).

Multiple BT determinations were also made in 18 pigs with saline administration prior to each BT. BT did not differ with section of mesentery ($p>0.05$), nor with time after laparotomy ($p>0.05$; Figure 5). Determination of mesenteric BT is therefore highly reproducible and not dependent on position or time after laparotomy, indicating that multiple BT determinations using this method are valid.

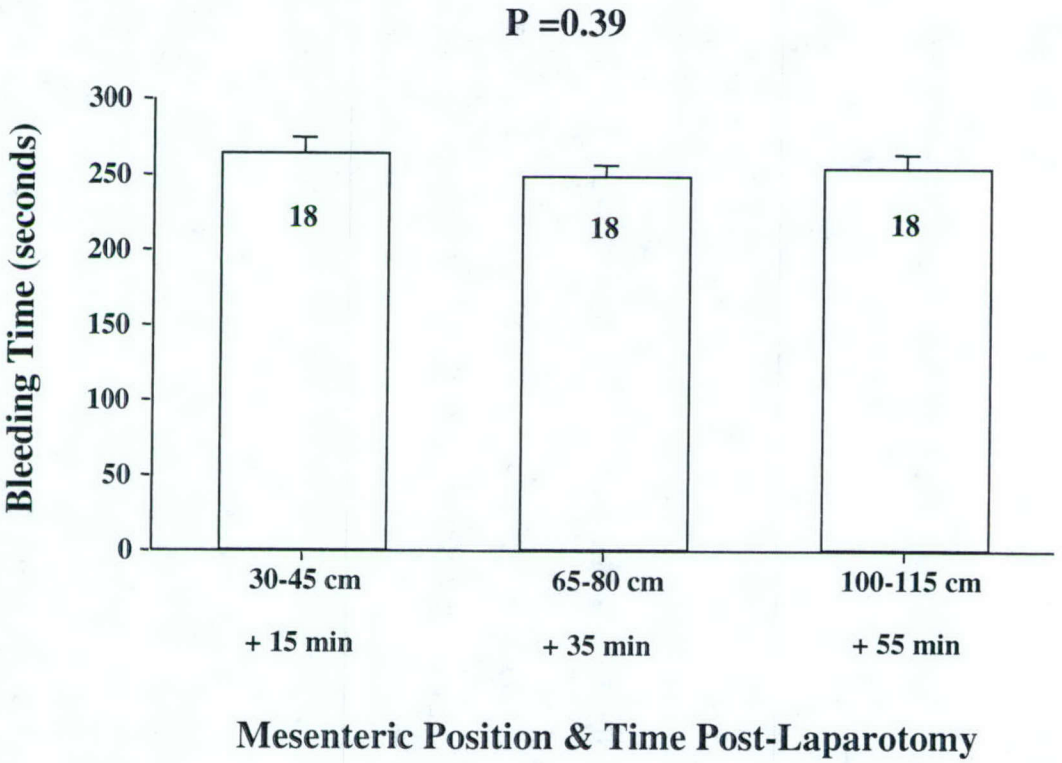


Figure 5. BT did not vary with mesenteric position. Distances are measured from the ileo-cecal junction.

Following determination of BT, a severe liver injury was performed. Through a series of range-finding experiments, we determined that excision of approximately 40-45% of the left medial and left lateral lobes was likely to produce hemorrhage of a severity that resulted in death within a 2 hour observation period in approximately 50% of animals. Table 1 reports observations from a series of 10 pigs which received this degree of liver injury. This liver injury resulted in death of 60% of the pigs during the 2 hour observation period. Those pigs that survived did not differ from non-survivors in

either the volume of blood lost or in the number or size of veins cut. Non-survivors did have a greater percent of their left lateral liver lobe removed (p=0.009). When all pigs were considered, 62% of the blood vessels cut were in the predefined small category, and this percentage differed (p<0.001) from those in the intermediate and large categories (23% and 16%, respectively).

Table 2. Characteristics of liver injury model in anesthetized swine.

Parameter	Survivors	Non-Survivors	Statistical Evaluation
N	4	6	
% Survival	40	60	P=0.53
Survival time (min)	120	61.7±19.9	P=0.004
Blood loss (gms)	824±106	884±103	P=0.71
% Lt. medial lobe cut	39.0±18.0	42.9±2.3	P=0.24
% Lt. lateral lobe cut	37.9±2.0	54.7±3.7	P=0.009
# Small veins cut/%**	26	36	
# Intermediate veins cut/%	12	11	P=0.61
# Large veins cut/%	6	10	(Chi-Square)

**The total number of veins cut was 101. Therefore, the number of veins and the percent of the total veins are almost identical. In one of the 10 pigs (a non-survivor), the number of veins was not evaluated. Numbers of veins are absolute values across 9 pigs.

Drug Treatments

FVIIa-PCPS. Because *in vitro* studies had shown a profound magnification of the efficacy of FVIIa by the addition of PCPS [29, 30], we initially sought to determine whether this same amplification of the FVIIa effect could be observed *in vivo*. Initial doses of FVIIa used in combination with the first two pigs (#24 and #55) were therefore minute (0.45 and 1.27 μg/kg for low and high doses, respectively) compared with previous doses of rFVIIa administered to pigs (≥ 90 μg/kg). Subsequently, the largest doses of FVIIa available from Haematologic Technologies (17.5 and 35 μg/kg) were administered to a third pig (#53). Selected raw hematological and BT data are presented

in Table 3. Administration of FVIIa with PCPS at concentrations previously found to be efficacious in *in vitro* studies [29] did not seem to decrease BT or enhance coagulation function in these three animals. Furthermore, administration of FVIIa-PCPS did not produce alterations in MAP. Following liver injury, blood loss and survival times were: 897.3 g and 120 min for pig #24; 1372.5 g and 29 min for pig #55; and, 864.2 g and 112 min for pig #53. There was no evidence of DIC on later histological examination.

Table 3. BT and coagulation parameters following infusion of various doses of FVIIa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury).

Pig #	Time Point	FVIIa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
24	T1	89.3	105	11.3	16.2	119.8	13.64	205	255
24	T2	136.1	102	10.5	16.7	114.3	14.40	190	312
24	T3	181.5	106	9.8	16	114.1	19.44	241	188
24	T4	116.6	110	10.3	16	122.7	45.62	331	---
55	T1	102.3	108	11.3	15.5	130.2	4.97	354	319
55	T2	125.4	125	9.9	15.6	134.1	5.12	335	261
55	T3	183.1	108	9.6	15.8	134.3	9.07	348	231
55	T4	120.8	107	10	14.9	122.3	33.52	303	---
53	T1	96.3	106	10.9	16	120.4	7.2	185	268
53	T2	594.9	104	9.4	15.9	123.4	15.3	181	292
53	T3	596.9	96	9.4	16.3	117.1	16.6	139	333
53	T4	406.3	114	9.6	16.2	115.7	83.2	118	---

Although we wished to increase the dose of FVIIa to levels closer to those previously shown to alter coagulation function in pigs [23], this was not possible due to the inability of Haematologic Technologies to purify and supply the required quantities of FVIIa. We also explored the possibility of obtaining a donation of recombinant FVIIa from NovoNordisk for testing purposes; such a donation was declined by NovoNordisk. At this point, the decision was made to drop continued testing of FVIIa in this study as we had no source for the drug at the levels required.

PCPS alone. In one pig (#25), PCPS was given by itself. Raw hematological and BT data are presented in Table 4. Infusion of PCPS did not seem to alter either coagulation function or BT. Furthermore, PCPS infusion did not alter MAP. There were no remarkable pathological findings in this pig.

Table 4. BT and coagulation parameters following infusion of two doses of PCPS (14 and 40 nmoles). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury).

Pig #	Time Point	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
25	T1	99	10.7	15.4	174.6	21.62	237	263
25	T2	96	10.7	15.1	170.8	22.9	206	292
25	T3	91	10.6	16.2	135.5	18.46	190	333
25	T4	99	10.3	15.5	153.1	50.15	266	---

FXa-PCPS. In the first series of FXa-PCPS experiments, the drug was administered according to the timeline depicted in Figure 2. Raw hematological and BT data are presented in Table 5. Pig #20 received doses of FXa-PCPS in a total volume of 8 ml; this infusion resulted in profound hypotension following the first dose and death immediately following the second. Following consultation with Dr. Michael Nesheim, it was then decided that the drug must be infused in larger volumes (1 ml/kg body weight); this was done in all subsequent pigs. Infusion of FXa-PCPS in these doses did not seem to enhance coagulation function or BT; in fact, BT and several coagulation factors (e.g., ACT, PT) were prolonged following administration of these doses.

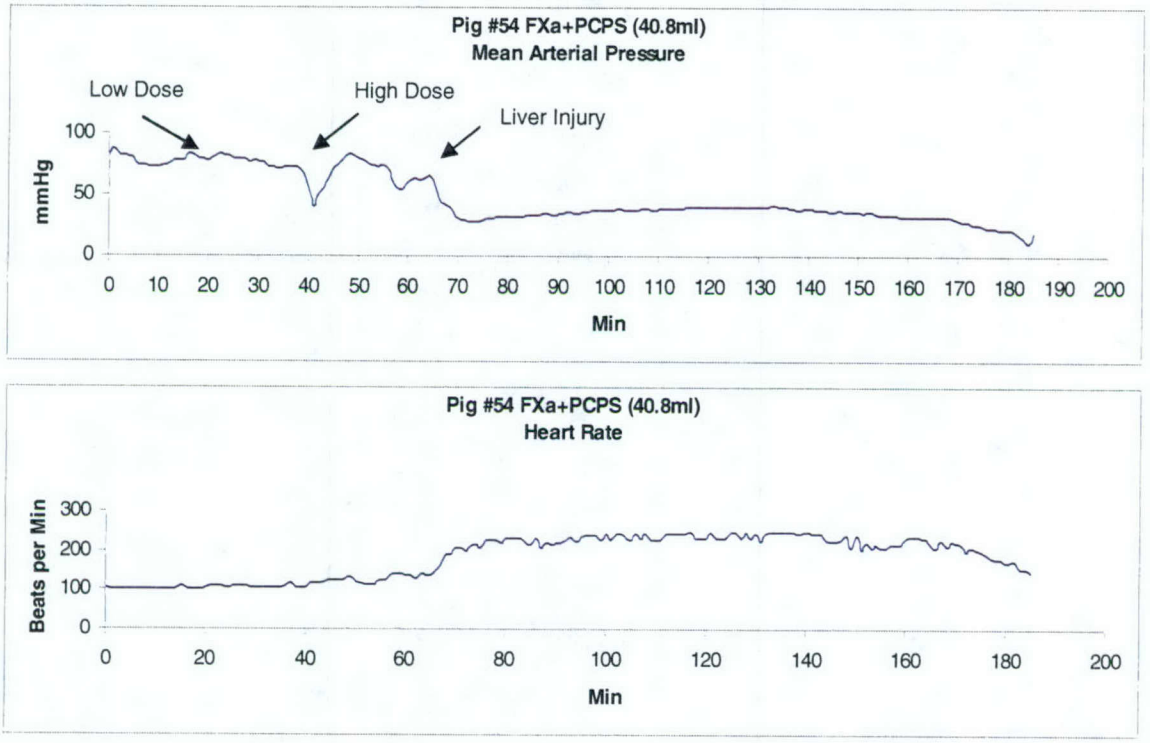
Table 5. BT and coagulation parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 2. BT not measured at T4 (post-liver injury). *No data collected due to death.

Pig #	Time Point	FXa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
20	T1	151.8	94	10.0	15.4	124.4	10.35	255	208
20	T2	18.6	170	14.3	17.0	No clot	1253.42	190	>600
20*	T3	---	---	---	---	---	---	---	---
20*	T4	---	---	---	---	---	---	---	---
21	T1	103.2	103	10.1	15.9	124.4	13.99	274	217
21	T2	103.6	101	10.2	15.2	129.1	28.35	290	227
21	T3	100.8	138	10.8	15.3	102.7	415.39	173	521
21	T4	86.0	107	11.3	16	103.6	105.37	206	---
54	T1	130.4	94	10.5	14.9	120.4	8.69	392	416.7
54	T2	132.6	93	10.5	14.8	124.2	17.39	408	526
54	T3	126.3	105	10.3	14.8	127.6	130.57	441	220.5
54	T4	104.4	92	10.7	14.9	154.7	115.66	402	---
62	T1	130.5	96	10.4	15.8	142.0	8.187	256	204
62	T2	113.4	103	10.4	15.4	127.0	11.411	299	505
62	T3	115.4	95	10.5	15.6	126.2	16.896	252	236
62	T4	105.0	108	10.1	15.5	153.2	54.730	258	---

Administration of these doses of FXa-PCPS also produced dose-dependent hypotensive responses, as shown in Figure 6. In performing BT measurements, we noticed that bleeding from the severed mesenteric vessels would decrease as MAP decreased and would increase markedly as MAP increased as a result of compensatory cardiovascular reflexes. In several of these experiments, bleeding would subsequently continue unabated until our 10-min maximum BT.

Following liver injury, blood loss and survival times were: 624.2 g and 120 min for pig #21; 864.2 g and 112 min for pig #53; and, 1089.6 g and 120 min for pig #62. There were no remarkable pathological findings in these pigs.

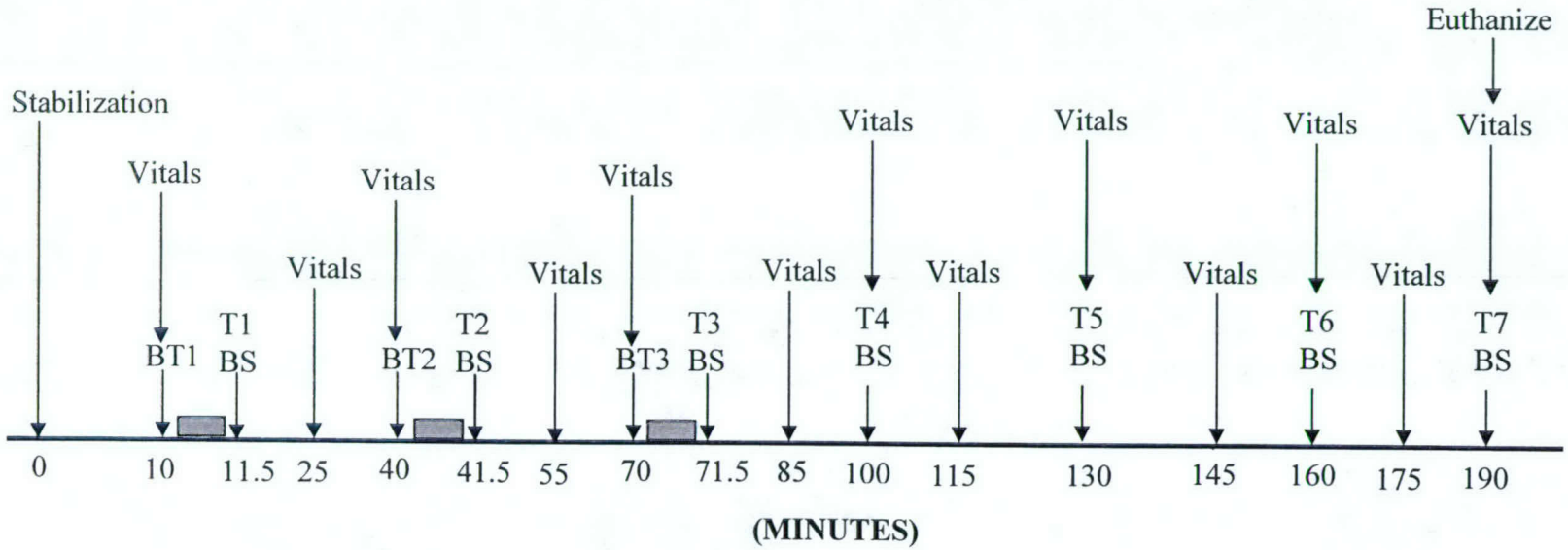
Figure 6. MAP and HR traces for a representative experiment. Following administration of the high dose in this pig, MAP decreased by 37 mm Hg.

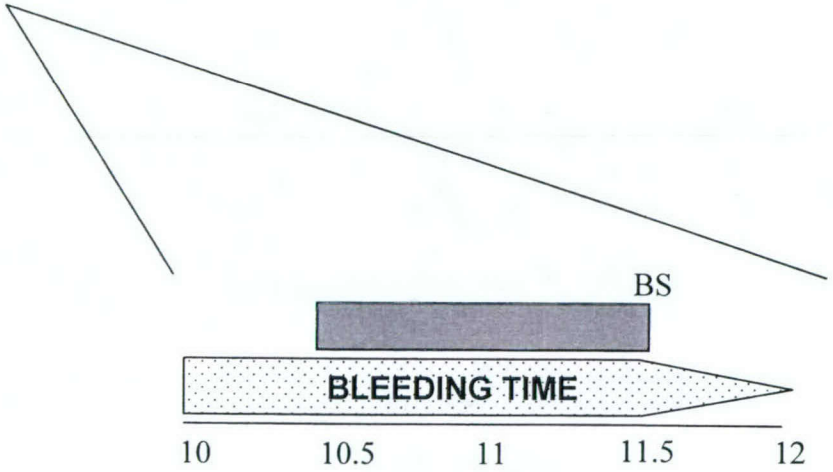


Following completion of these experiments, the decision was made to alter the timeline of subsequent experiments to maximize the chances of observing a beneficial effect on BT, as well as to allow collection of blood samples at a number of time points following drug infusion (instead of performance of a liver injury). In the remaining 5 pigs which received FXa-PCPS, the timeline depicted in Figure 7 was used. One feature of this timeline was the beginning of BT determination 30 sec before drug infusion, to take into account the extremely short half-life of FXa (<15 sec). In two of these pigs (#102 and #103), the low dose of drug (2.5 pmoles FXa/4.0 nmoles PCPS) was given first and was followed by the high dose (25.5 pmoles FXa/40 nmoles PCPS); this order was reversed in the remaining three pigs (#101, #118 and #121). This was done to ensure that

administration of the low dose did not consume substrate (i.e., fibrinogen) to a point which would not allow full expression of any beneficial hemostatic effect from the high dose. Again, this was to maximize the possibility of observing any reduction in BT.

Figure 7. Timeline for Last 5 FXa-PCPS Experiments





Drug Infusion
BT Bleeding Time
BS Arterial Blood Sample
* Taken before death (MAP≤20)

Raw hematological and BT data from this second group of five pigs treated with FXa-PCPS are found in Table 6. In each of the three pigs for which a complete set of BT's was collected, FXa-PCPS markedly increased BT. Pigs #118 and 121 died approximately 5 minutes after infusion of the high dose of FXa-PCPS, an occurrence which falsely lowered their BT's following FXa-PCPS (due to pronounced hypotension; see below). In each of the 5 pigs, infusion of the high dose of FXa-PCPS produced increases in PT, aPTT, and TAT, while decreasing Fib-C and PLT levels.

TEG was also measured in these 5 pigs (Table 7). In pigs #102 and 103, the low dose (administered first) did not seem to profoundly alter TEG parameters from baseline (T2 vs. T1). Subsequent administration of the high dose of FXA-PCPS did not seem to immediately (T3) alter R values, although all other parameters were profoundly changed, such that the MaxVel of clot formation and MA (a measure of clot strength) were both decreased. Thirty minutes after drug infusion (T4), R and K were both lengthened while MA, tMA, and MaxVel were all still depressed. In the only pig that survived administration of the high dose first (i.e., #101), R value was immediately depressed upon infusion (T2) but MaxVel was increased. Subsequently, R, K and tMA were lengthened while α-angle, MA, and MaxVel were lengthened. From these preliminary data and the BT, PT, and aPTT data, we concluded that administration of FXa-PCPS at doses sufficient to produce alterations in coagulation function negatively impacted the ability to clot.

Table 6. BT and coagulation parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 7.

Pig #	Time Point	FXa (%)	ACT (sec)	PT (sec)	aPTT (sec)	Fib-C (mg/dl)	TAT (µg/l)	Plt (10^3/mm^3)	BT (sec)
102	T1	128.5	104	10.4	15.5	165.1	5.64	159	296
102	T2	120.7	95	10.5	15.5	164.2	11.24	154	536
102	T3	105.9	90	11.4	17.1	90.5	266.45	46	>600
102	T4	104.8	143	13.3	18.1	83.0	199.23	136	---
102	T5	98.0	125	13.9	18.1	83.0	141.41	132	---
102	T6	101.0	120	13.6	18.1	83.6	114.48	139	---
102	T7	98.7	121	13.2	17.9	88.1	3.47	130	---
103	T1	149.9	97	9.6	15.8	178.6	11.99	431	480
103	T2	148.1	93	9.5	15.6	178.5	20.62	402	413
103	T3	128.0	96	12.5	16.5	83.0	194.16	230	>600
103	T4	140.2	123	12.1	17.5	83.00	176.35	360	---
103	T5	133.7	111	11.5	17.6	83.00	162.23	332	---
103	T6	128.6	113	11.4	17.0	93.10	132.85	344	---
103	T7	133.6	108	11.3	17.6	93.40	115.13	366	---
101	T1	105.9	95	10.9	15.8	122.8	4.15	297	320
101	T2	108.7	88	12.1	15.7	83.0	239.6	372	372
101	T3	97.6	101	13.3	16.6	83.0	154.0	275	477
101	T4	98.0	116	13.3	16.9	83.0	85.9	288	---
101	T5	83.5	108	13.2	17.7	83.0	28.0	291	---
101	T6	95.3	107	12.7	16.7	83.0	21.1	294	---
101	T7	99.8	103	12.5	16.7	86.9	17.9	276	---
118	T1	149.5	84	9.9	15.7	112.7	6.8	188	246
118	T2	150.7	75	11.8	15.8	83.0	180.2	79	145
118	T3	---	---	---	---	---	---	---	---
118	T4	---	---	---	---	---	---	---	---
118	T5	---	---	---	---	---	---	---	---
118	T6	---	---	---	---	---	---	---	---
118	T7	---	---	---	---	---	---	---	---
121	T1	119.0	105	10.7	15.3	96.7	4.7	350	230
121	T2	108.4	242	35.5	28.4	no clot	267.1	68	136
121	T3	---	---	---	---	---	---	---	---
121	T4	---	---	---	---	---	---	---	---
121	T5	---	---	---	---	---	---	---	---
121	T6	---	---	---	---	---	---	---	---
121	T7	---	---	---	---	---	---	---	---

Table 7. TEG parameters following infusion of various doses of FXa-PCPS (doses found in Table 1). Experimental time points according to timeline shown in Figure 7.

Pig #	Time Point	R (min)	K (min)	α angle (degrees)	MA (mm)	tMA (min)	MaxVel (mm/min)	tMaxVel (min)
102	T1	2.4	0.9	78.1	65.4	12.4	23.08	3.33
102	T2	1.4	0.8	79.1	67.8	11.7	26.8	2.22
102	T3	2.2		20.3	9.6	10.6	1.76	1.13
102	T4	10.8	2.7	54.3	50.5	25.9	7.74	13.17
102	T5	5.2	1.7	65.5	52.8	18.9	12.28	6.53
102	T6	4.3	1.6	67.6	54.1	16.4	13.66	5.61
102	T7	4	1.4	70.2	54.8	14.8	14.55	5.17
103	T1	2.1	0.9	77.3	67.7	12.8	25.44	3.17
103	T2	1.6	0.8	79.2	70.8	11.4	28.95	2.42
103	T3	1.7	2.1	59.9	35.1	7.7	11.22	3.39
103	T4	6.4	1.7	63.2	53.7	18.6	13.85	7.92
103	T5	3.7	1.4	70.1	57.6	17	15.28	4.78
103	T6	3.7	1.4	68.1	59	17.8	16.22	4.97
103	T7	3.2	1.2	71.6	57.4	16.1	16.69	4.25
101	T1	1.8	0.8	79.5	67.5	12.9	31.03	2.47
101	T2	0.9	1.6	70.4	65.7	13.4	18.12	2.81
101	T3	2.7	1.6	67.1	59.9	17.8	12.58	3.92
101	T4	3.2	1.3	69.8	54.4	15.6	15.44	4.11
101	T5	3	1.3	68.6	58.1	17.4	15.71	4.25
101	T6	2.5	1.1	73.5	59.1	15.7	18.55	3.39
101	T7	2.8	1.2	73.3	61.1	14.2	18.11	3.69
118	T1	2.3	1.1	75.4	66.5	15.2	22.48	3.31
118	T2	1.8	2.1	60.6	57.4	12.8	11.28	3.42
118	T3	---	---	---	---	---	---	---
118	T4	---	---	---	---	---	---	---
118	T5	---	---	---	---	---	---	---
118	T6	---	---	---	---	---	---	---
118	T7	---	---	---	---	---	---	---
121	T1	2.3	0.8	78.8	66.4	12.9	28.67	3.06
121	T2	39.7	9.8	12.8	16.4	57.7	1.32	43.72
121	T3	---	---	---	---	---	---	---
121	T4	---	---	---	---	---	---	---
121	T5	---	---	---	---	---	---	---
121	T6	---	---	---	---	---	---	---
121	T7	---	---	---	---	---	---	---

Figure 7 depicts MAP and HR from a representative experiment in which the low dose of FXa-PCPS was administered first. Figure 8 shows MAP and HR from the only pig that survived infusion of the high dose of FXa-PCPS first (pig #101). In all 3 pigs that survived infusion of the high dose of FXa-PCPS, a profound hypotensive response was observed with this dose, with maximal decreases in MAP of 43 (pig #102), 41 (pig #103), and 42 mm Hg (pig #101). In these pigs, infusion of the lower dose of FXa-PCPS resulted in decreases in MAP of 17, 10, and 14 mm Hg (for pigs 102, 103, and 101, respectively). Figure 9 depicts MAP and HR during an experiment in which administration of the high dose of FXa-PCPS resulted in lethal hypotension.

Again, there was no evidence of DIC or microthrombi formation in these animals.

Figure 7. MAP and HR from a representative experiment in which the low dose of FXa-PCPS was administered first, followed by the high dose.

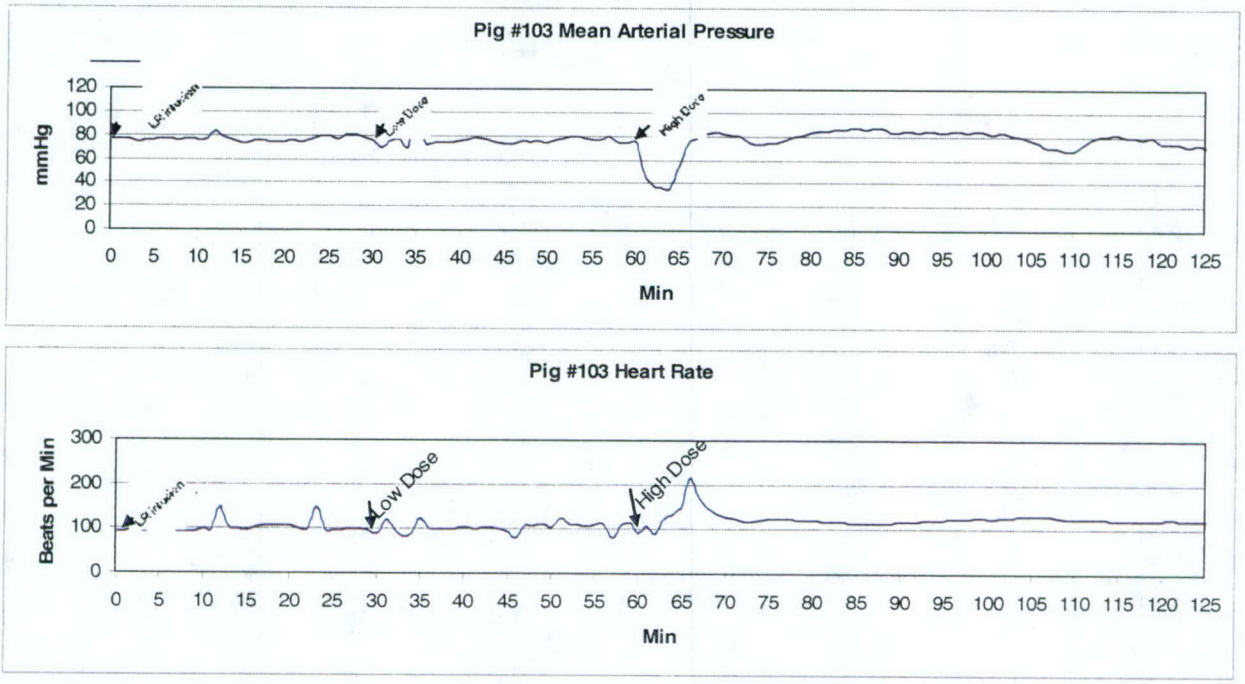
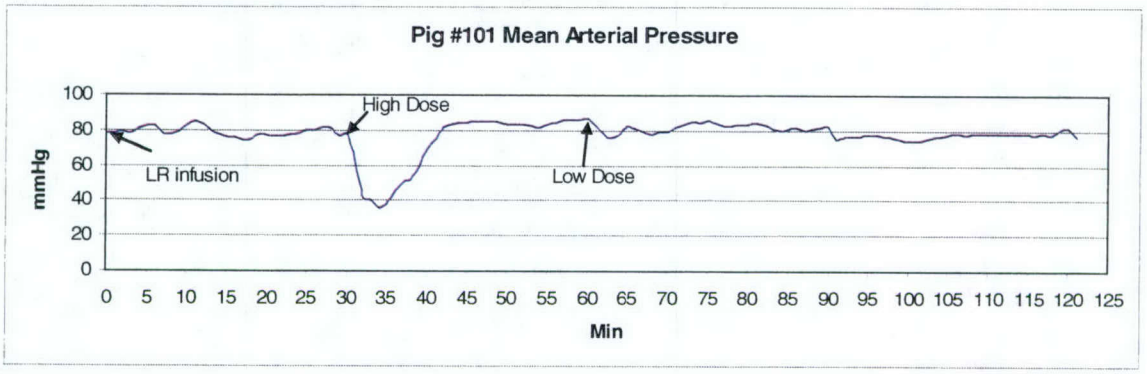


Figure 8. MAP and HR from pig #101, in which the high dose of FXa-PCPS was administered first, followed by the low dose.



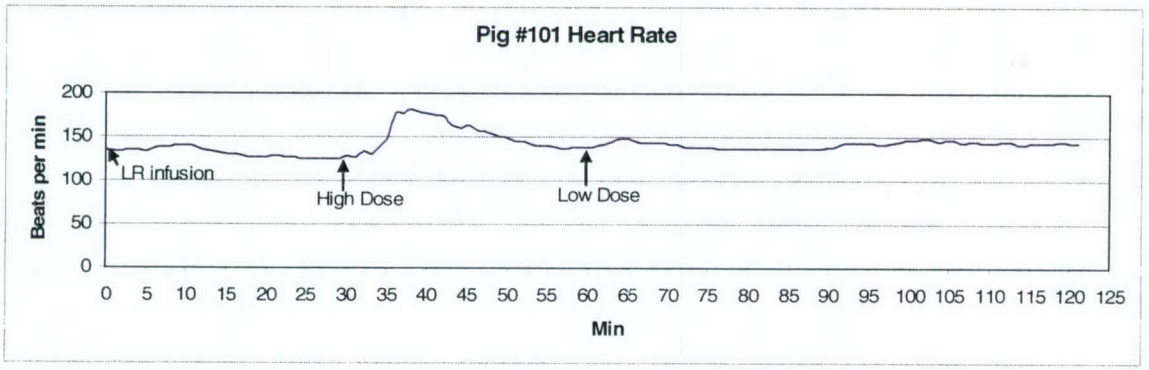
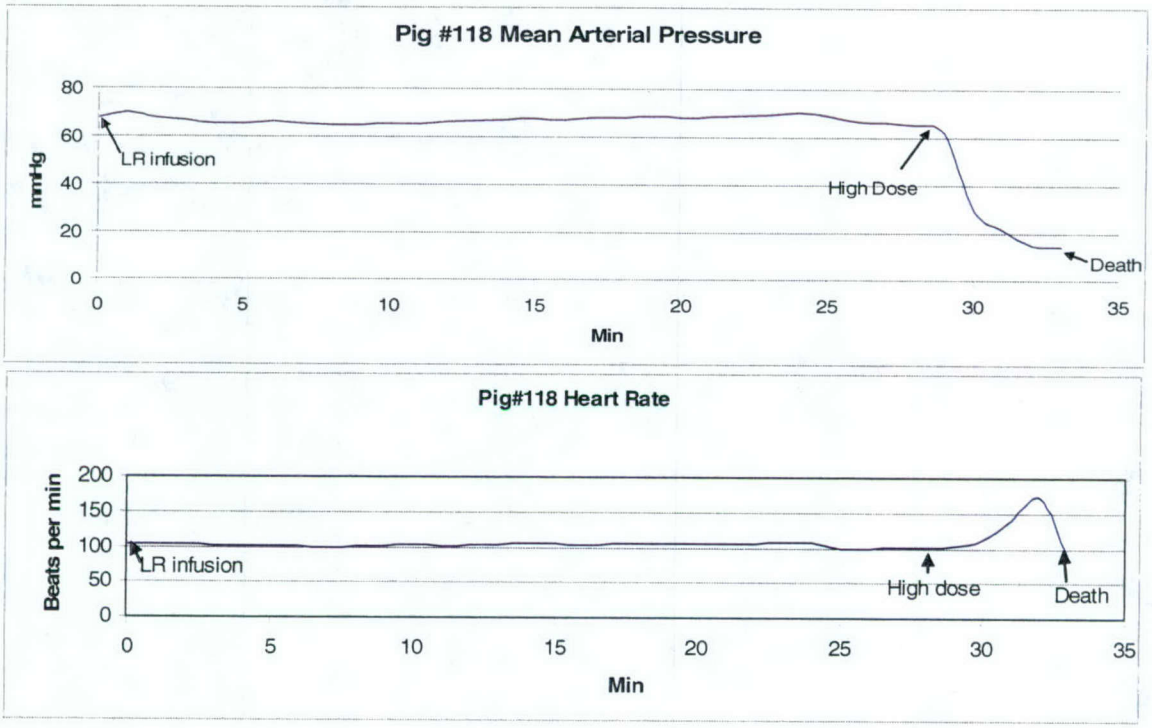


Figure 9. MAP and HR from a representative experiment in which administration of the high dose of FXa-PCPS produced lethal hypotension.



DISCUSSION

In this preliminary series of experiments, we were successful in developing a novel animal model in which intravascular hemostatic agents could be screened to determine suitability for further testing for possible treatment of traumatic injury. This model allows the determination of hemostatic responses to escalating doses of drug, an approach that has been used previously in this laboratory [23]. In this earlier study, BT was determined by injury to either the ear or the spleen of swine. Both methods have been associated with logistical problems, including the requirement to maintain ear temperature constant, the inability to perform multiple (>2) BT due to the low surface area available for injury sites, the inability to perform more than 2 replicates at each BT measurement, and the contractile nature of the spleen. The use of the mesenteric BT

seems to obviate many of these problems and appears to be more reproducible than previous methods. Because of the evident reproducibility between anatomical locations in the gut, it is apparent that one can make BT determinations with multiple doses of hemostatic drug. In further support of this contention, blood flow has been shown to be comparable among the porcine duodenum, jejunum, and ileum [36].

The primary blood supply to the porcine intestine is via the cranial mesenteric artery [37]. Within the mesenteries of the small intestine the arteries and veins lie adjacent to each other with a small amount of connective tissue between them [37]. Hence, BT determination using the current technique differs from other methods inasmuch as the mesenteric BT relies on bleeding from small arteries and veins rather than capillary bleeding. Despite this caveat, it is clear that the mesenteric BT performed herein was sensitive to alterations in coagulation function as BT was correlated with alterations in ACT produced by infusion of heparin and protamine. Furthermore, inhibition of coagulation function induced by FXa-PCPS infusion and reflected in alterations in TEG and other coagulation parameters were associated with prolongations of BT. It should be noted that this laboratory is currently performing experiments that will simultaneously determine both mesenteric and splenic BT for validation purposes.

Another feature of this animal model is that it allows, within the same animal, an initial determination of how pretreatment with a drug of interest affects a traumatic uncontrolled hemorrhage. In previous work, intravenous agents have been tested in a swine model (originally developed for testing the efficacy of hemostatic dressings) that included complete transection of major veins (>10 mm in diameter) of the liver [15, 16, 21, 23]. While entirely appropriate for testing hemostatic dressings, it is questionable

whether such a model is appropriate for testing intravenous hemostatic drugs, as it is improbable that such drugs could be highly effective in such a situation (i.e., big holes in big vessels). Additionally, this previously-used model required aggressive resuscitation to produce exsanguinating hemorrhage leading to death. Again, such resuscitation is not entirely appropriate for testing intravascular agents as it leads to dilution of the intravascular agent under study. Because of these considerations, we sought to develop a severe liver injury model that 1) was exsanguinating without either heparinization or aggressive resuscitation; and, 2) did not involve transection of the major veins of the liver, but instead produced more diffuse bleeding from smaller vessels. Additionally, we desired an injury that produced a mortality rate of approximately 50% in order to discern whether the drug under study had either beneficial or detrimental effects on survival. The selective excision of approximately 45% of both the left medial and lateral lobes described herein achieved these goals as it produced 60% mortality within 2 hours via transection of predominantly small ($\leq$ 4 mm diameter) hepatic veins. Furthermore, this mortality rate was achieved without using either aggressive resuscitation or heparinization. We therefore believe that this model is a reasonable one for testing of intravenous hemostatic agents.

Having developed a model, we then sought to determine the efficacy of either FVIIa-PCPS or FXa-PCPS to reduce blood loss and enhance coagulation function. The available doses of FVIIa-PCPS did not seem to alter either hemostatic function or BT in these limited trials, despite the fact that such concentrations were effective in enhancing coagulation function *in vitro* [29]. These doses of FVIIa, however, were much lower than those previously shown to be efficacious for recombinant FVIIa in swine [23].

Whether the addition of PCPS to higher doses of FVIIa would shift the dose-response curve for FVIIa to the left (demonstrating increased potency) is a question that remains unanswered due to lack of the drug in these amounts.

The question of whether FXa-PCPS might be useful in traumatic indications has been answered to our satisfaction by these pilot experiments. Administration of FXa-PCPS produced detrimental effects on coagulation function (as assessed by TEG and standard coagulation tests) and on BT. The range of doses used in these preliminary experiments had previously been found to be efficacious in both normal and hemophilic dogs with cuticular bleeding [32, 33, 38, 39]. Furthermore, similar doses had also been used in rabbits [31, 39] and nonhuman primates [38, 39]. In these experiments in pigs, however, these doses appeared to produce a consumptive coagulopathy, as suggested by the observed decreases in fibrinogen and platelets and associated detriments in coagulation function.

Additionally, we observed that administration of doses required to alter coagulation function also produced profound hypotension; although the decrease of blood pressure appeared to be dependent on dose, we were unable to find a dose in these pilot experiments that provided beneficial effects on coagulation function without producing unacceptable levels of hypotension or coagulopathy. During experiments, we observed that bleeding from the mesenteric vessels used for BT determination seemed to decrease as blood pressure decreased, but then increased again as blood pressure increased. It is therefore possible that cessation of cuticular bleeding in dogs could have also been associated with a decrease in blood pressure, although this was not measured [32, 33]. Dr. Michael Nesheim also stated that a hypotensive response was often seen in nonhuman

primates, although this has not been published (personal communication). Perhaps this profound hypotension should have been expected on infusion of FXa-PCPS, as elevation of FXa levels should increase systemic levels of thrombin (as suggested by the observed increases in TAT), which has previously been shown to produce vasodilation and profound hypotension [40]. Regardless, the profound hypotension induced by FXa-PCPS is not acceptable for treatment of patients who have suffered traumatic hemorrhage and may already be hypotensive.

To conclude, we have successfully developed a novel swine model for preliminary screening of intravenous hemostatic drugs. Using this model, we tested the ability of FXa-PCPS to decrease BT and enhance coagulation function. Rather than providing beneficial effects, FXa-PCPS actually induced coagulopathy and unacceptable decreases in blood pressure. Based on these preliminary results, we conclude that further testing of this drug for the indication of traumatic bleeding is unwarranted.

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